

Thaumatin-like protein in kiwifruit

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Abstract: A 21-kDa protein extracted from woody tissue underlying the pedicel of kiwifruit (*Actinidia deliciosa* var *deliciosa* [A Chev] Liang and Ferguson) was purified to homogeneity by cation exchange, gel filtration, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on a 15% gel and electro-transfer onto a polyvinylidene difluoride (PVDF) membrane, followed by excision of the band of interest. Amino terminal sequence analysis of this band revealed a high degree of homology with basic endochitinase (EC 3.2.1.14) in orange and thaumatin-like proteins in tobacco, barley, maize and tomato. This is the first record of a thaumatin-like protein in a subtropical berryfruit. Potential implications of this finding and the need for future research are discussed.

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Keywords: thaumatin; chitinase; kiwifruit; N-terminal amino acid sequence

INTRODUCTION

Pathogenesis-related (PR) proteins are a family of acid-soluble, heat- and protease-resistant proteins that accumulate in plants in response to pathogen infection or environmental stress. They were first discovered in tobacco (*Nicotiana tabacum*) plants reacting to tobacco mosaic virus infection, but closely related proteins have been subsequently detected in many plant species and are thought to play a role in host resistance.¹ PR proteins have been characterized into five classes, three of which have been identified as β -1,3-glucanases (Class 2), chitinases (Class 3) and the thaumatin-like (TL) proteins (Class 5). The latter purportedly inhibit fungal pathogens through non-enzymic membrane permeabilization. To date, PR-5 proteins have been described in cereals,^{2–4} sunflower,⁵ flax⁶ *Solanaceous* species,^{7,8} lupin⁹ and cherry.¹⁰ The presence of chitinases and TL proteins in kiwifruit, an important horticultural export crop in New Zealand, Chile, Italy and in other countries, was investigated in this study.

MATERIALS AND METHODS

Reagents

All chemicals were Analar Reagent quality, from Sigma, BDH, Serva, Bio-Rad Laboratories, May and Baker, or Ajax Chemicals. Unless otherwise stated, water was obtained from a Milli-Q mixed bed ion exchange filter (Millipore) using a reverse osmosis processed feed stock.

Electrophoresis

The Laemmli¹¹ system, adapted for use with slab gels,

was used for denaturing polyacrylamide gel electrophoresis (SDS-PAGE). Isoelectric focusing (IEF), application of a 0.01% (w/v) glycol chitin overlay gel to the IEF gel once focusing was complete, and staining of the overlay gel with 0.01% (w/v) Calcofluor white in 500 mM Tris-HCl, pH 8.9, to visualise chitinase activity were carried out as described by Wurms *et al.*¹² SDS and IEF gels were silver-stained according to the method of Merrill *et al.*¹³

Western blotting

Western blotting was carried out after SDS-PAGE according to Wurms *et al.*¹² except that a mixture of two primary antibodies (both diluted 500-fold in phosphate buffered saline –0.05% Tween 20) was applied to the nitrocellulose membrane after the blocking step. The primary antibodies (gifts from DANISCO Biotechnology, Copenhagen) were raised to one basic (CH2) and one acidic (SP) chitinase from sugar beet. In initial trials, the sugar beet antibodies were applied singly to Western blots to analyse individual binding patterns.

Protein purification

Export quality (102 g mean weight) kiwifruit (*Actinidia deliciosa* var *deliciosa* cv Hayward [A Chev] C. F Liang et A R Ferguson), harvested on 23 May 1994 from a commercial orchard in Levin, New Zealand, were cured in open trays for one week at 20 °C following pedicel removal, then stored at 0(±0.3) °C for 12 weeks. Stem plugs (comprising the wound surface created by pedicel removal and the underlying pin of sclerified tissue) were excised from healthy fruit, snap

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frozen in liquid nitrogen and extracted as described previously,¹⁴ except that extraction buffer included 10 mM sodium tetrathionate, 0.02% (w/v) Triton X-100 and 1% (w/v) polyvinylpyrrolidone, and that 1 mM dithiothreitol replaced 0.2% (w/v) sodium dithionite. One part (by weight) stem plug tissue was extracted in three volumes of buffer. Extract (32 ml) was dialysed against approximately 250 ml of 20 mM sodium acetate (NaOAc) buffer, pH 5 for 12 h at 5 °C with stirring. It was lyophilized, reconstituted in 3.3 ml of Milli-Q water, filtered through a 0.45 µm Sartorius disposable filter and exchanged into 20 mM NaOAc buffer, pH 5 using a "Slide-a-Lyzer" dialysis cassette (10 000 molecular weight cut-off, Lab supply Pierce).

A 3-ml aliquot of the resultant dialysate was injected onto a Bio-Rad Econo-Pac S cation exchange column equilibrated with 20 mM NaOAc, pH 5. Adsorbed protein fractions were eluted after 7 min washing with a 0–1 M linear gradient of NaCl in 20 mM NaOAc over 50 min. Protein elution was monitored by optical density in a LKB Uvicord, model 4700. The flow rate was 1 ml min⁻¹ and fractions were collected every min.

A chitinase assay¹⁵ modified for use in Petri dishes (as described below), was used to identify active fractions containing chitinases, which could be expected to co-purify with TL proteins during cation exchange and gel filtration.^{2,16}

Fractions with chitinase activity, which had bound to the ion exchange column and eluted at 31–36 min, were pooled, freeze dried, reconstituted in 170 µl of Milli-Q water and centrifuged at 13 000 *g* for 5 min. A 75-µl aliquot of this pooled supernatant was passed through a Phenomenex BioSep SEC-S3000 (250 × 10 mm) HPLC gel filtration column, equilibrated with 20 mM NaOAc buffer, pH 5, using a Hewlett Packard 1050 Ti-Series HPLC. Fractions, collected at 0.5-min intervals, were eluted with the same buffer at a rate of 1 ml min⁻¹. Proteins in these HPLC fractions were monitored by measuring absorbance at 218 and 280 nm using a Hewlett Packard Multiple Wavelength detector. The fractions were lyophilized and stored at –20 °C until required.

Chitinases and thaumatin in the SEC-S3000 fraction eluted at 14 min were subsequently separated by SDS-PAGE. For larger-scale preparations this separation could be achieved by hydrophobic chromatography.^{2,16} Approximately 20 µg of lyophilized protein from the active gel filtration fraction was redissolved in 10 µl of water, followed by addition of 10 µl of 2 × sample buffer (0.125 M Tris-HCl, pH 6.8, 4% (w/v) SDS, 20% glycerol (v/v), 10% (v/v) 2-mercaptoethanol, 0.05% (w/v) bromophenol blue). After 2 min boiling, the sample was applied to a polyacrylamide resolving gel (T=15%, C=2.7%) with a stacking gel (T=4%, C=2.7%) on top. A boiled 20-µl aliquot of low molecular weight SDS-PAGE standards (Sigma) was also applied directly to the gel. The gel was electrophoresed at 100 V, using Laemmli¹¹ electrode buffer, until the bromophenol dye front had moved

into the resolving gel and then at 120 V until the dye had reached the bottom of the gel.

A polyvinylidene difluoride (PVDF) membrane (Applied Biosystems) and the SDS-PAGE gel were equilibrated in blotting buffer (0.01 M 3-[cyclohexylamino]-1-propanesulfonic acid, 10% methanol) followed by electrophoretic transfer at 90 V for 40 min. The PVDF membrane was stained for 1–2 min in 0.1% (w/v) Ponceau S, 1% (v/v) acetic acid, and destained with water, then air dried and stored at –20 °C until sequencing. The band of interest was cut out from the blot using a scalpel blade and was placed in a glass cartridge. *N*-terminal sequence analysis was carried out using an Applied Biosystems gas phase sequencer (Model 470A) equipped with an on-line phenylthiohydantoin amino acid analyser (Model 120A), and chemicals and programme supplied by the manufacturer. The sequence was compared with other *N*-terminal amino acid sequences using the Basic Local Alignment Search Tool (BLAST) on the Internet.

Chitinase Petri dish assay

Agar powder (Oxoid) (6 g) was added to 390 ml of pH 5 citrate phosphate (McIlvaine) buffer, 0.05 M citric acid/0.10 M disodium phosphate, containing 0.02% (w/v) azide. The suspension was heated to boiling in a microwave to dissolve the agar, then 4 ml of 1% (w/v) pH 4 glycol chitin (prepared as described by Trudel and Asselin)¹⁵ was added after 10 min cooling, and the mixture was poured into 8.5-cm diameter Petri plates (20 ml per plate). Once the agar had set, seven evenly spaced 5-mm diameter wells were punched into the agar, using a cork borer attached to a vacuum pump. The plates were stored in sealed plastic bags at 4 °C until required. Test extract (30 µl) was added to each well, and the plate was sealed with Parafilm. After 5 h at 37 °C, 10 ml of 0.01% (w/v) Calcofluor white M2R (Fluorescent brightener 28, Sigma) in 500 mM tris(hydroxymethyl)aminomethane hydrochloric acid (Tris-HCl) buffer, pH 8.9 was added. After 10 min, the plate was rinsed in reverse osmosis water several times and left covered with water for 15 min. The water was drained, and the plate viewed under long wavelength UV light (350 nm). Zones in which the glycol chitin had been degraded appeared as dark circular areas, while the remainder of the plate fluoresced blue/white due to the stain attached to the glycol chitin.

RESULTS AND DISCUSSION

The SEC-3000 gel filtration fraction which eluted at 14 min contained at least three proteins (Fig 1). The predominant protein in this fraction had a basic pI of >8.3 (Fig 1), and a molecular mass of 21 kDa (Fig 2). These characteristics are common to TL proteins which have low molecular masses (20–25 kDa) and basic pI values,^{3,6,17,18} although some acidic isoforms are known.^{5,19} The predominant protein also showed some chitinase activity in a glycol chitin overlay gel

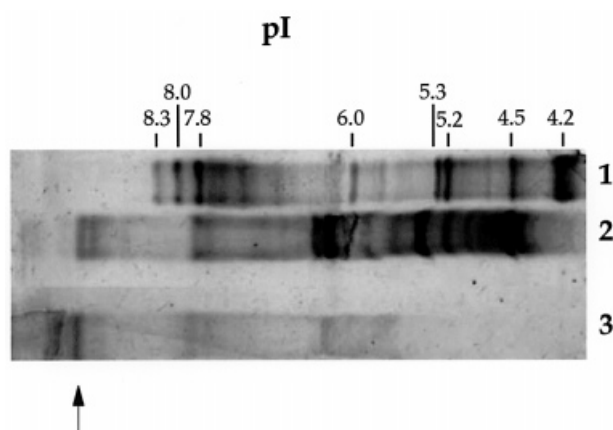


Figure 1. Silver-stained, pH 3–10, IEF polyacrylamide gel of proteins from an extract of an uninoculated, healthy, cured kiwifruit stem plug. Lane 1, pI standards (1 μ l, SERVA Cat No 39211); lane 2, 4 μ l of crude kiwifruit stem plug extract; lane 3, 4 μ l of semi-purified stem plug extract recovered from the central peak (14 min) of gel filtration chromatography on a SEC-S3000 HPLC column following adsorption to an Econo-Pac S cation exchange column. The predominant protein in lane 3 is arrowed. The 'band' in the centre of this lane was created by residual protein that had not diffused out of the applicator strip.

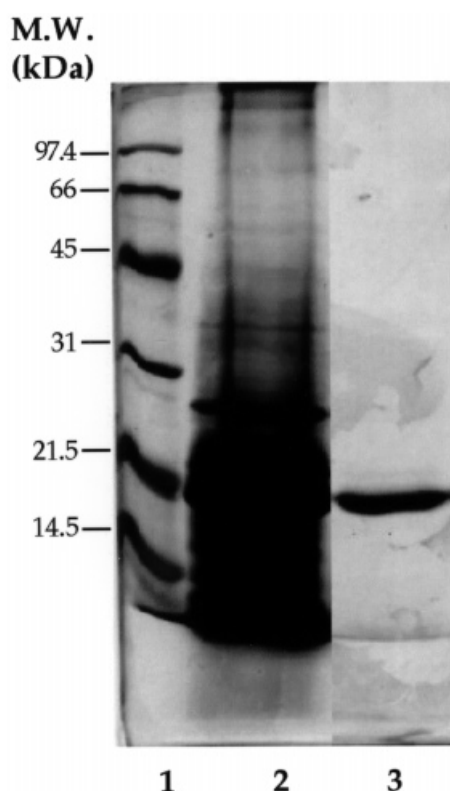


Figure 2. Silver-stained SDS-PAGE of total proteins in crude and semi-purified cured, uninoculated, healthy kiwifruit stem plug extract. Lane 1, molecular weight standards; lane 2, crude extract; lane 3, $\approx$ 5 μ g of semi-purified stem plug extract after separation on cation exchange and gel filtration columns.

which was a blueprint of the IEF gel shown in Fig 1 (results not presented because of poor resolution). Based on binding specificity to affinity-purified polyclonal antibodies ('CH2' and 'SP') raised to sugar beet

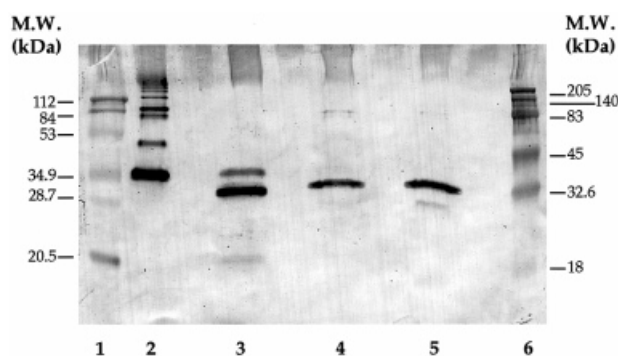


Figure 3. Western blot analysis of proteins in various chitinase preparations treated with CH2 and SP antibodies raised against sugar beet basic and acidic chitinase. Lanes 1 and 6, prestained molecular weight standards (Bio-Rad); lane 2, partially purified bean chitinase (the bean chitinase was supplied by D Greenwood, and had undergone cation exchange chromatography); lane 3, cured, uninoculated, healthy kiwifruit stem plug extract which was partially purified through cation exchange and gel filtration; lane 4, crude, uncured kiwifruit stem plug extract; lane 5, crude, cured kiwifruit stem plug extract. Each lane contained $\approx$ 5 μ g of protein. CH2 and SP antibodies were diluted 500-fold in PBS-Tween.

chitinases, two of the proteins in the SEC-3000 fraction eluting at 14 min appeared to be chitinases (Fig 3). However, CH2 antibody did not bind to the 21.5-kDa protein and whilst SP binding was observed on occasion, the binding was weak and inconsistent (results not shown).

The predominant protein in the 14 min SEC-3000 fraction was separated from other proteins by SDS-PAGE, followed by electro-transfer onto a PVDF membrane, protein staining and excision of the band of interest. N-terminal sequencing of this band produced a single clean sequence free of background contamination (Knight C, pers comm.). Comparisons with other published sequences showed that the protein was highly homologous with a citrus endo-chitinase N-terminal amino acid sequence and with numerous thaumatin-like proteins (Table 1 and Fig 4). These homologies suggest that the kiwifruit protein is a member of the PR-5 family and may exhibit chitinase activity. This novel finding opens a whole host of possibilities for future research, including whether this compound influences disease resistance and taste in kiwifruit.

Most TL proteins are thought to be antifungal, but their function has not been clearly elucidated.^{8,20} Inhibition of pathogen enzyme systems has been suggested as a mode of action, because many TL proteins are highly homologous to a protein which inhibits both trypsin and α -amylase, although not all members of the PR-5 family exhibit such enzyme-inhibitory activity despite sequence homology.^{3,7} A more likely mechanism for fungistasis is non-enzymic membrane permeabilization, since maize zeamatin and several other TL proteins induce hyphal rupture of various fungi.^{2,16,21} Curing of the kiwifruit stem wound has been associated with the rapid collapse of subsequently applied spores of *Botrytis cinerea*.²² The

Table 1. Sequence homology between a kiwifruit pathogenesis-related (PR) protein and other proteins from the PR-3 and PR-5 families

Protein	Source	Accession number	Sequence identity with SEC-3000 fraction eluted at 14 min	Reference
Thaumatococcus protein	Kiwifruit	P81370	100%	This paper
Basic endochitinase	Orange	1881844	82%	23
Thaumatococcus protein	Tobacco	P13046	72%	24
Thaumatococcus protein	Grape	2213852	72%	25
Osmotin-like protein	Arabidopsis	P50700	72%	Unpublished
Osmotin-like protein	Grape	E300865	72%	Unpublished
Thaumatococcus protein	Tobacco	P07052	65%	17
Thaumatococcus protein	Barley	P33044	58%	3

underlying mechanism has yet to be identified. It will be interesting to discover whether curing induces production of the kiwifruit TL protein. Chitinase activity is not a characteristic of TL proteins described to date.^{2,3,16} Although chitinase activity was present in the partially purified SEC-3000 14 min fraction and the N-terminal sequence of the purified protein was homologous with an endochitinase, further work is required to determine whether kiwifruit TL protein exhibits chitinase activity. This would require purification of sufficient quantities of the protein to test for antifungal activity and to establish the mode of action. An important first step would be to find a suitable method of assaying TL activity. In other studies this has been achieved by measuring the unusual property of TL proteins to act synergistically with nikkomycin to inhibit the growth of *Candida albicans*.² Assaying for membrane permeabilization as well as a range of

enzyme inhibitory activities and the use of in vitro antifungal assays should help to establish the mode of action of the TL protein.

Another important aspect of future research would be to determine the possible influence of the TL protein on flavour in kiwifruit. Thaumatinins are often sweet-tasting, although not all TL proteins share this property.^{7,17,21} Antigenic sites in thaumatococcus in some cases appear to be the same sites which interact with human sweet taste receptors.⁷ If the TL protein is present in the edible portions of kiwifruit, it may have an effect on fruit flavour. Antibodies to TL could be useful in routine assessment of TL levels. Antibodies could also be used in immuno-labelling studies to visualise location of the protein within the fruit.

Hence, further research on kiwifruit TL proteins is warranted and will promote an understanding of the role of PR-5 proteins in plants.

K/F	A	T	F	N	I	I	N	N	C	P	F	T	V	W	A	A	A	V	P	G	G	G	K	R	L	D	R	G	Q
OCHIT	A	T	F	D	I	R	N	N	X	P	Y	T	V	W	A	A	A	V	P	G	G	G	R	R	L	D	R	G	
PRR1	A	T	F	D	I	V	N	K	C	T	Y	T	V	W	A	A	A	S	P	G	G	G	R	R	L	D	S	G	Q
GRATH	A	T	F	D	I	L	N	K	C	T	Y	T	V	W	A	A	A	S	P	G	G	G	R	R	L	D	S	G	Q
ARATH	A	T	F	E	I	L	N	Q	C	S	Y	T	V	W	A	A	A	S	P	G	G	G	R	R	L	D	A	G	Q
GRAOS	A	T	F	N	I	Q	N	H	C	S	Y	T	V	W	A	A	A	V	P	G	G	G	M	Q	L	G	S	S	Q
THRR	A	T	I	T	V	V	N	R	C	S	Y	T	V	W	P	G	A	L	P	G	G	G	V	R	L	D	P	G	Q
PRR2	A	T	F	D	I	V	N	Q	C	T	Y	T	V	W	A	A	A	S	P	G	G	G	R	Q	L	N	S	G	Q
consensus:	A	T	-	-	+	-	N	-	-	-	+	T	V	W	+	+	A	-	P	G	G	G	-	+	L	-	-	G	Q

Figure 4. N-terminal amino acid sequence comparisons between a kiwifruit pathogenesis-related (PR) protein and other proteins from the PR-3 and PR-5 families. K/F, thaumatococcus protein from kiwifruit stem plug extract; OCHIT, endochitinase from orange (accession number 1881844); PRR1, tobacco thaumatococcus protein PRR1 (accession number p13046); GRATH, grape thaumatococcus protein (accession number 2213852); ARATH, osmotin-like protein from Arabidopsis (accession number p50700); GRAOS, grape osmotin-like protein (accession number e300865); THRR, barley thaumatococcus protein fragment THRR (accession number p33044); PRR2, tobacco thaumatococcus protein PRR2 (accession number p07052). Amino acids from position 1 up to a maximum position of 29 in K/F, OCHIT and THRR are aligned with amino acids in positions 23 to 51 in ARATH, 25 to 53 in GRATH and GRAOS, and 26 to 54 in PRR1 and PRR2. Positions with identical residues in all proteins are marked with the letter of the appropriate residue, conserved positions (amino acid substitutions that are unlikely to alter function) with + signs, and non-conserved replacements with a – sign.

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